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PRINCIPAL INVESTIGATOR: Adam Mueller

CONTRACTING ORGANIZATION: University of Virginia
Charlottesville, VA 22908

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Adam Mueller

E-Mail: acm4d@virginia.edu

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14. ABSTRACT

We have identified the miR-99 family of microRNAs as being downregulated in more advanced cancer cell lines, having expression that inversely correlates with radiation resistance. The miR-99 family is transiently upregulated following DNA damage, and when expressed exogenously can sensitize cells to ionizing radiation. Through its target, SNF2H, the miR-99 family can reduce BRCA1 foci formation at sites of DNA damage, and reduce the rate of DNA repair following damage by IR. Furthermore, exogenous miR-99a and miR-100 can reduce the overall efficiency of double strand break repair by both homologous recombination as well as non-homologous end joining. When exposed to ionizing radiation, the transient up regulation of the miR-99 family leads to cells less efficiently recruiting BRCA1 to DNA damage foci after repeated exposure to radiation, an effect that is abrogated by antisense inhibition of miR-99 induction. This downregulation of the efficiency of DNA repair may represent a mechanism by which normal cells abort repair and survival after repeated mutagenic events, and whose loss confers resistance to DNA damaging radiation and chemotherapy in advanced cancer cells.

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Introduction:

O-GlcNAc and Emi1 in genomic instability of breast cancer.

One of the key steps in initiation and progression of breast cancer is the loss of control over DNA replication and the cell cycle that lead to genetic instability and aneuploidy. Gene amplification and transcriptional misregulation resulting from this instability represent some of the last steps in the formation of malignant neoplasias. O-linked N-acetyl glucosamine is a post-translational protein modification similar in many respects to protein phosphorylation in the number of targets that are modified and the variety of cellular pathways it modulates(1). An offshoot of the glycolysis pathway, it is thought to act as a nutrient sensor, its addition modulated by the availability of glucose. Other groups have shown that mis-regulation of O-GlcNAc can lead to faulty cell cycle regulation and aneuploidy in HeLa(2). Further defects in control of the cell cycle and DNA repair may act synergistically with O-GlcNAc regulatory defects to cause malignant transformation. We have shown that depletion of Early Mitotic Inhibitor 1(Emi1) leads to cell cycle defects and re-replication in normal breast epithelial cells(3). We have proposed to examine whether siRNA knockdown of Emi1 along with O-GlcNAc Transferase(OGT) and O-GlcNAcase(NCOAT) can act synergistically to cause large scale re-replication, aneuploidy and eventually lead to malignant transformation in breast epithelial cells.

Supplemental Aim: Determining the role of microRNAs in the response to DNA damage in breast cancer.

A great deal of recent attention has been placed on the role of microRNAs in cancer, especially in regards to cell survival and proliferation. MicroRNAs are a recently discovered class of small RNA molecules that post-transcriptionally regulate the expression of a plethora of genes and cellular pathways. They play a role in the differentiation and function of numerous cell types across species, and some have been shown to dramatically affect cell survival(4,5). They have been shown to regulate both p53(6) and the estrogen receptor(7), and have the potential to contribute to the progression of breast cancer. They also represent excellent therapeutic targets, as they can now be readily introduced as well as inhibited both in cells in culture as well as in vivo in animals or patients(8). The function and regulation of miRNAs are collectively a focus of this lab, as is the role of genomic instability in cancer, and the examination of their potential role in the response of breast cancer to DNA damage ties into the goals of this project. We intend to identify microRNAs whose expression is modulated in response to DNA damage, as well as those differentially expressed between cancers with varying resistance to DNA damaging radiotherapy and chemotherapy, in order to to identify microRNAs that may be able to

enhance normal breast epithelial cells ability to cope with re-replication stress as well as modulate cancer cells ability to withstand treatments. Expression of microRNAs targeting key components of the DNA damage response, cell cycle and DNA replication could have a significant effect on the level of response a breast cancer has to a treatment regimen, and introducing exogenous or blocking expressed miRNAs could confer potentially chemo or radiosensitivity to treatment refractory cases of cancer.

Body:

Task 1: Testing checkpoint activation, cell cycle effect and re-replication after co-depleting Emi1 and OGT or NCOAT

siRNA depletion of OGT blocks Emi1 induced DNA re-replication in some breast cancer cell lines.

Our first goal is to determine if Emi1 knockdown in conjunction with mis-regulation of O-GlcNAc post-translational modification of proteins can act synergistically to cause re-replication. We have obtained antibodies as well as siRNA oligos against OGT, NCOAT and Emi1. We treated MCF7 and Sk-Br-3 breast cancer cells as well as MCF10a breast mammary epithelial cell lines with 20nM siRNA oligos targeting various combinations of OGT, NCOAT, Emi1 and GL2 firefly luciferase using Invitrogen Lipofectamine RNAimax transfection reagent. The cells were collected 72 hours post-transfection and analyzed by propidium iodide flow assisted cell sorting(FACS) to determine the cell cycle profile and whether re-replication was occurring in these cells. We generated whole cell lysates from these samples as well to perform western analysis in order to confirm knockdown as well as examine cell cycle and checkpoint markers.

In MCF10a and MCF7 we observed a substantial population of cells with greater than 4N DNA content, characteristic of re-replication, with Emi1 treatment. This re-replication was largely abrogated by treatment with siRNA against OGT(fig 1a, 1b). We were only able to achieve a small degree of re-replication with SKBR3, making it difficult to determine the effect of OGT RNAi, but this effect was slightly enhanced by NCOAT knockdown(not shown). As shown in figure 1c, we achieved efficient knockdown of OGT, and the effect of OGT knockdown on re-replication was not through rescue of geminin levels, which are reduced due to increased APC activity. Interestingly, Cyclin A levels were reduced with OGT RNAi, this is noteworthy because co-depletion of geminin and Cyclin A are sufficient to cause re-replication in HeLa cells which are resistant to geminin induced re-replication.

siRNA depletion of OGT blocks MLN4924 induced DNA re-replication.

MLN4924 is a small molecule inhibitor that prevents an activating neddylation modification of cullins, that has been found to cause apoptosis in cancer cells through s-phase arrest or re-replication(9).

Within 24 hours of treatment with MLN4924, we have been able to see significant re-replication in a number of cell lines, making it an efficient tool in studying re-replication mechanisms. We treated cells with siRNA against OGT for 48 hours prior to treating with MLN4924, and prepared the samples for PI FACS. As shown, OGT RNAi was sufficient to cause a substantial reduction in the number of re-replicating cells with MLN4924 treatment(Fig. 2a).

OGT inhibition with ST060266 can block re-replication by MLN4924

We obtained a small molecule inhibitor of OGT, ST060266 to determine if chemical inhibition of OGT could prevent re-replication initiated by MLN4924. We pretreated cells for 12 hours with ST060266 prior to the addition of MLN4924. As shown, there were substantially fewer re-replicating cells with OGT inhibition than with DMSO(fig2b).

OGT inhibition has a modest effect on re-replication induced following release from thymidine block.

In order to determine if OGT could inhibit re-replication through a mechanism other than blocking entry into S-phase, we treated cells for 12 hours with 2mM thymidine for 12 hours, GM for 12 hours, thymidine for 12 hours prior to releasing. We used HCT116 colon cancer cells due to their relative ease of synchronizing. 12 hours prior to release, we treated with Alloxan, an inhibitor of OGT. Cells were treated with MLN4924 8 hours prior to release, and released into MLN4924 and nocodazole. Cells were collected for FACS over 24 hours. As shown, at 24 hours, there was a modest decrease in the amount of MLN4924 induced re-replication that occurred when Alloxan was added(fig2c). This indicates that DNA re-replication may require the action of OGT following entry into S-phase.

OGT knockdown halts DNA replication.

We performed a BrdU ELISA to determine the rate of DNA synthesis following Emi1 and OGT RNAi. 72 hours following treatment with siRNA in 96 well plates, we examined BrdU incorporation. As shown, by 72 hours, Emi1 knockdown has resulted in an abrogation of BrdU incorporation likely secondary to checkpoint activation. A similarly dramatic reduction is seen with OGT inhibition(fig3a), even though the cells have a normal FACS profile. This would seem to indicate that O-GlcNAc modification is required for normal DNA synthesis as well as re-replication, and may regulate multiple phases of the cell cycle.

PCNA is candidate for modification with O-GlcNAc

To identify substrates of OGT, we performed pull-down experiments with wheat germ agglutinin, a lectin that binds to O-GlcNAcated proteins. Cells were treated with Alloxan or DMSO for 24 hours prior to lysis. Lysates were incubated with WGA before washing and boiling with SDS loading buffer. Western blotting of the pull-downs were then performed for Cdt1, MCM2, CDK2 and PCNA, all proteins which are crucial for DNA replication. Of these, PCNA was found to associate with WGA, making it a likely candidate for regulation by OGT.

Identifying microRNAs that regulate the DNA damage response that are differentially expressed between breast cancers

In order to examine microRNAs that were involved in regulating the DNA damage response, we performed microarrays to detect miRNA expression in MCF7 and SKBR3 breast cancer cell lines following 24 hours of 5gy IR treatment. Mir99a was one of the microRNAs that was identified to be downregulated 2 fold in MCF7. Additionally, its basal expression level was found to be 2.2 fold higher in MCF7 than SKBR3. We examined the expression level of miR99a by qRT-PCR in MCF10a, SKBR3 breast cell lines, as well as p53+/+ and -/- HCT116 cells. We found expression to be significantly lower among the p53 mutant cell lines, however, we could not find any difference in expression following DNA damage(fig4a). Our lab has the additional interest in determining microRNAs that are differentially regulated over the course of prostate cancer progression, and miR99a was identified as a microRNA that was downregulated 5 fold in the androgen independent cell line C4-2, vs. the androgen dependent progenitor cell line LnCAP. We decided to examine the potential role of miR99a in the DNA damage response in both breast and prostate cancer cell lines.

Cdc25a is a target of miR-99a

Cdc25a was found to be a predicted target of miR99a by multiple algorithms. The main function reported for Cdc25a is that its degradation following DNA damage inhibits S-phase progression as part of the cell's adaptive response(Bartek and Lukas 2001). Targeting of Cdc25a by microRNAs could potentially regulate the kinetics and magnitude of the intra-s-phase checkpoint. To determine whether it is a target, we introduced exogenous miR99a into C4-2 cells and performed sucrose gradient fractionation to purify polyribosome and monoribosome associated mRNAs. When miR99a was introduced, we found Cdc25 shifted from the polyribosome fraction to the monosome fraction indicating that translation was inhibited(fig 4b, 6a). Additionally, when we introduce exogenous miR-99a into

cells, we see a reduction in Cdc25a protein level to below that of irradiated cells(Fig 6c). Finally, we cloned the Cdc25a 3'UTR into a luciferase reporter vector and found that exogenous miR-99a could reduce luciferase expression by 50%(Fig. 6d), an effect that was negated by mutation of the predicted miR-99a target site(shown Fig 6a). Taken together, these data show that Cdc25a is a target of miR-99a.

miR99a decreases 3H thymidine incorporation following DNA damage

To determine whether miR99a can alter the DNA damage response, we examined whether its introduction could alter tritium labeled thymidine incorporation following 10gy gamma irradiation in MCF10a cells. Prior to radiation treatment, cells were labeled with 14C-thymidine for 24 hours to normalize to cell number and basal growth rate. A 15 minute 3H-thymidine pulse was performed prior to each time point following radiation. We found a modest reduction in 3H-thymidine incorporation at the 30 minute time point(fig4c). Additionally, we performed a dose titration of ionizing radiation in LnCAP and C4-2 cells in which miR99a had been introduced. With exogenous miR99a, we found a reduction in thymidine incorporation in both cell lines 2 hr following IR, particularly in C4-2 which has low endogenous miR99a expression(fig5a,b), and robustly decreased 60' following 5gy IR in C4-2 cells(Fig. 6b).

miR99a sensitizes C4-2 cells to DNA damage

To determine whether introduction of miR99a can confer modulate a cells sensitivity to DNA damage, we introduced it into LnCAP and C4-2 cells which express it at different endogenous levels, and performed clonogenic survival assays following a dose titration of gamma irradiation. We found that in LnCAP cells, there was little effect on cell survival with the introduction of more miR99a(Fig. 5c), which was already highly expressed, however in C4-2, exogenous miR99a caused a marked decrease in cell survival(Fig. 5d), decreasing the colony numbers at the 2.5 and 5 gy doses to below the those seen in LnCAP, which was more sensitive than the control C4-2 cells. Interestingly, we see a similar sensitization with siRNA knockdown of Cdc25a(Fig. 6f), indicating this may be an important target of miR-99a in mediating radiation sensitivity of cancer cells. This data indicates that miR99a has the ability to modulate cells immediate response to DNA damage, as well as their long term survival following exposure to radiation, which may prove to be useful as a diagnostic indicator as well as perhaps a therapeutic target.

The miR-99 family is transiently induced following ionizing radiation and can modulate the DNA damage response by regulating SNF2H

See appendix for paper in preparation describing this work.

Key accomplishments:

- Shown OGT inhibition blocks siEmi1 induced re-replication
- Determined that chemical inhibition of OGT by ST060266 modestly inhibits re-replication
- Determined that chemical inhibition of OGT cannot block MLN4924 induced re-replication when added following thymidine block and release.
- Showed 48 hour treatment with siEmi1 as well as siOGT dramatically reduces BrdU incorporation.
- Identified miRNA's regulated by DNA damage in breast cancer cell lines MCF7 and Sk-Br-3 by microarray
- Observed Cdc25a becomes monosome associated following miR99a introduction
- Confirmed a decrease in Cdc25a protein after introduction of miR-99a
- Observed a decrease in Cdc25a 3'UTR coupled luciferase activity by miR-99a in a target site specific manner
- Observed a decrease in post irradiation 3H-Thymidine incorporation in breast and prostate cancer cells following miR99a introduction
- Identified miR-99a as having an anti-survival and radiosensitizing effect on breast cancer cells following DNA damage
- Cdc25a siRNA radiosensitizes cells to IR
- Expression of miR-99a is transiently upregulated following DNA damage
- miR-99a can sensitize C4-2 cancer cells to DNA damage
- miR-99a and siRNA targeting SNF2H can reduce BRCA1 foci formation at sites of DNA damage
- miR-99a reduction of BRCA1 foci formation is dependent on SNF2H
- miR-99a alters the kinetics of DNA repair following ionizing radiation, reducing the rate of repair in a SNF2H dependent manner
- miR-99a/miR-100 reduce the efficiency of both homologous recombination as well as non-homologous end joining after introduction of double strand breaks
- Blocking the upregulation of miR-99a/miR-100 following DNA damage blocks the observed decrease in DNA repair foci formation following multiple rounds of ionizing radiation

Reportable Outcomes:

Abstract submitted for 2010 Cold Spring Harbor Mechanisms & Models of Cancer 2010 Meeting:

miR99a, a microRNA that is repressed during prostate cancer progression, can modulate the cellular response to DNA damage by regulating Cdc25a synthesis.

Adam C Mueller[1], Dandan Sun[1], Anindya Dutta[1]
[1]University of Virginia, Biochemistry and Molecular Genetics, Charlottesville, VA, 22908

Abstract submitted for 2011 Keystone Meeting on Genomic Instability and DNA repair:

Downregulation of Mir-99a during progression of prostate cancer decreases checkpoint activity and makes the cancer cells resistant to DNA damage.
Authors: A Mueller; D Sun; A Dutta
[1]University of Virginia, Biochemistry and Molecular Genetics, Charlottesville, VA, 22908

Paper in preparation:

The miR-99 family is transiently induced following ionizing radiation and can modulate the DNA damage response by regulating SNF2H

Adam C Mueller[1], Dandan Sun[1], Anindya Dutta[1]
[1]University of Virginia, Biochemistry and Molecular Genetics, Charlottesville, VA, 22908

Conclusions:

O-GlcNAc and Emi1 in genomic instability of breast cancer.

We were able to show that siRNA depletion of OGT was able to block DNA rereplication induced by knockdown of Emi1. Additionally, we were able to show that re-replication induced by the cullin inhibitor MLN4924 was also reduced by siRNA of OGT. Chemical inhibition of OGT by ST060266 appeared to reduce this re-replication as well, but we were unable to determine that OGT activity was in fact reduced in vivo by this somewhat uncharacterized inhibitor. Short pretreatment with alloxan could not block rereplication induced by MLN4924, indicating that longer inhibition of OGT activity is necessary in order to block re-replication. We have also seen a dramatic reduction in BrdU incorporation with OGT inhibition, indicating its function is required for general DNA synthesis as well as re-replication to occur. The growth inhibition observed with OGT knockdown seems to point towards a more generalized effect on the cell cycle, where cells may be arrested throughout the cell cycle, which would appear to be normal by FACS analysis, but be unable to continue to divide. We also identified PCNA as a substrate for OGT, which being a critical component of the replication machinery would be a likely target for OGT regulation of DNA synthesis. The sheer number of substrates modified by OGT may make it difficult to determine exactly which one or many are responsible for this effect, but it may

be quite useful to potentially target the enzyme itself as highly metabolically active and rapidly replicating tumors may be susceptible to this observed reduction in growth following OGT inhibition.

Determining the role of microRNAs in the response to DNA damage in breast cancer.

We have identified a microRNA that is downregulated in more advanced breast and prostate cancer cell lines that appears to play a role in the DNA damage response. We have found that when exogenous miR99a is introduced into cells, Cdc25a shifts from the polysome to monosome fraction indicating its translation is being repressed. Expression of miR-99a reduces luciferase activity of a Cdc25a 3' UTR reporter, which is rescued by mutation of the miR-99a target site, validating Cdc25a is a direct target of miR-99a. Additionally introduction of miR99a causes a further decrease in 3H-thymidine incorporation following irradiation of breast and prostate cancer cells. Introduction of miR99a into a cell line where its expression is typically low causes a decrease in its ability to form colonies following treatment with ionizing radiation.

The miR-99 family is transiently induced following ionizing radiation and can modulate the DNA damage response by regulating SNF2H

We had previously identified SNF2H as a target of miR-99a, and in prostate cancer was responsible for the role of miR-99a in decreasing PSA expression. Recent studies have shown SNF2H to be required for efficient recruitment of DNA repair proteins to double strand break sites. We found that miR-99a could decrease recruitment of BRCA1 to ionizing radiation induced DNA repair foci, and that this was dependent on downregulation of SNF2H. Furthermore we observed a decrease in the rate of DNA repair by comet assay and a decrease in the efficiency of both homologous recombination and non homologous end joining type DNA repair in the presence of exogenous miR-99a and miR-100. Finally, having observed a transient upregulation of miR-99a/miR-100 following IR, we were able to show that this upregulation decreased the efficiency of BRCA1 recruitment to DNA damage sites after multiple rounds of ionizing radiation, and efficient recruitment could be rescued by blocking miR-99a/miR-100 induction following irradiation. This induction may represent a mechanism to prevent cells from attempting repair and survival following multiple mutagenic insults, a mechanism which is lost in cancer cells to allow them to become resistant to DNA damaging chemotherapy and radiotherapy. While we examined miR-99a/miR-100 loss in the context of advanced cancers becoming radioresistant, it would be of interest to determine whether inappropriate overexpression of miR-99a/miR-100 is seen in BRCA1 WT cancers, as their interference with BRCA1 function through SNF2H downregulation may play a different role in the developmental niche which breast cancer arises.

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Appendices:

The miR-99 family is transiently induced following ionizing radiation and can modulate the DNA damage response by regulating SNF2H

Abstract

Chromatin remodeling factors are becoming known as crucial facilitators of recruitment of repair proteins to sites of DNA damage. Several different chromatin remodeling complexes have been recently discovered to be required for efficient homologous recombination as well as non-homologous end joining type repair to occur. SNF2H has recently been found to facilitate the loading of BRCA1 at sites of DNA damage so that repair may occur. In a screen for microRNAs that might modulate the DNA damage response, we found expression of the miR-99 family to be correlated with radiation sensitivity and transiently induced following radiation. We found that by reducing levels of SNF2H, the mIR-99 family reduced the efficiency of DNA repair following subsequent rounds of DNA damage and may act as a measure in preventing cells exposed to multiple rounds of DNA damage to carry on survival after repeated mutagenic events.

Introduction

Mammalian cells have developed multiple complex mechanisms for identifying and repairing damage to their genomes caused by a wide variety of DNA damage sources. Damage caused by UV, ionizing radiation, crosslinking and intercalating agents as well as errors experienced during replication require different mechanisms for signaling that damage has occurred and repairing the incurred lesions. The ability of normal cells to detect and repair DNA damage, as well as to initiate apoptosis when too much damage is experienced is crucial for maintaining genomic stability and preventing cancer from arising. Conversely, cancer cells ability to sustain and repair large quantities of DNA damage contributes to their frequently observed eventual resistance to DNA damaging chemotherapeutic and radiation treatment. Much work has shown that several histone modifications specifically designate sites of DNA damage, and are required for the recruitment of numerous repair proteins. In particular, phosphorylation of H2AX has been robustly shown to be required for DNA damage signaling and repair(Celeste et al., 2002), but there is a steadily growing body of evidence that indicates several other histone modifications including ubiquitination, methylation and acetylation occur that play a role in conjunction with as well as independent of pH2AX signaling in facilitating effective DNA repair(van Attikum and Gasser, 2005; van Attikum and Gasser, 2009).

As with transcription, it appears one of the functions of these histone markers is to recruit chromatin remodeling complexes in order to enable access of repair proteins to sites of DNA damage. ATP dependent chromatin remodeling complexes were initially implicated in DSB repair when the INO80 complex was found to be recruited to phosphorylated H2A in budding yeast, and required for efficient conversion of double strand breaks into single stranded DNA(Morrison et al., 2004; van Attikum et al., 2007; van Attikum et al., 2004). More recently, the SWI/SNF complex has been shown both to be recruited to pH2AX in conjunction with acetylation of histone H3, as well as required for maximal phosphorylation of H2AX(Lee et al., 2010).

Double strand break repair in mammalian cells typically occurs through two distinct pathways, homologous recombination or non-homologous end joining. Homologous recombination type repair is dependent on the recruitment of BRCA1 to double strand break sites through a complex signaling cascade downstream of phosphorylated H2AX. The protein MDC1 binds to pH2AX(Stewart et al., 2003) and recruits the ubiquitin ligase RNF8, which subsequently ubiquitilyates H2A and pH2AX(Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007). RNF168 is recruited to ubiquitinated histones and forms conjugated ubiquitin chains which are essential for the recruitment of BRCA1 containing HR complexes(Doil et al., 2009). Non-homologous end joining involves the binding of Ku proteins to double stranded ends of DNA at break sites(Roberts and Ramsden, 2007), and recruits DNA-PKcs and DNA ligase IV which then facilitate the ligation of the free ends(Mladenov and Iliakis, 2011). Both processes require the action of chromatin remodeling proteins for proper recruitment of their components to DNA damage sites(Lan et al., 2010) (Larsen et al., 2010).

Recently, the chromatin remodeling complex SNF2H has been implicated to be involved in both the HR and NHEJ DNA repair pathways. It was shown that following DNA damage, SNF2H was required for the efficient recruitment of Ku70/80 proteins to laser stripe induced DNA damage sites, and its depletion resulted in inefficient DSB repair by NHEJ(Lan et al., 2010). Interestingly, SNF2H was also shown to be required for BRCA1 recruitment to break sites in a pH2AX independent manner downstream of H2B ubiquitination by RNF20(Nakamura et al., 2011).

microRNAs are small 17-21 nucleotide RNA molecules that regulate expression of transcripts in metazoans in a sequence specific manner. microRNAs are crucial for development of higher organisms and each microRNA can regulate the expression of many proteins to enact complex changes in cellular phenotype. microRNAs have been found to be intimately involved in the differentiation of many tissue types during development(Bruno et al., 2011; Dey et al., 2011; Gagan et al., 2011). Additionally, in cancer, microRNAs are often misregulated, with certain miRNAs able to function as tumor suppressive and oncogenic factors(Lee and Dutta, 2007; Lee and Dutta, 2009).

In a screen for microRNAs that could modulate cells response to DNA damage, we came across a microRNA family that was upregulated following DNA damage and whose expression correlated with radiation sensitivity. We had characterized SNF2H to be a target of the miR-99 family(Sun et al., 2011), and wanted to examine whether miR-99 could decrease the efficiency of DNA repair by downregulating SNF2H. Interestingly, we found that the transient upregulation of the miR-99 family following radiation could decrease the efficiency of repair after multiple rounds of DNA damage, and may represent a switch by which cells are directed away from DNA repair after multiple mutagenic insults and towards cell death.

Results and discussion

miR99 family members downregulated in radioresistant cancer sensitize cells to DNA damage by ionizing radiation.

We were interested in examining microRNAs whose expression were misregulated in cancer and could alter the radiation sensitivity of cancer cells. We initially began by measuring differential microRNA expression in two breast cancer lines, MCF-7 and SK-BR-3 before and 24 hours following treatment with 5gy ionizing radiation. In MCF-7 we observed modest effects of radiation on the expression of microRNAs previously characterized as oncogenic such as miR-21(Papagiannakopoulos et al., 2008) as well as tumor suppressive such as the p53 regulated miR-34a (Chang et al., 2007; Raver-Shapira et al., 2007)(Table 1). Interestingly, we found that two microRNAs we had previously identified as differentially regulated between androgen dependent and androgen independent prostate cancer(Sun et al., 2011). miR-99a and miR-100 were both expressed at higher levels in MCF-7 cells as compared to SK-BR-3 and upregulated following IR. Confirmation by qPCR showed miR-99a and miR-100 to be 4 fold upregulated in MCF7 as compared to SK-BR-3(Fig. 1a). When examining the expression between LnCAP and C4-2 prostate cancer cell lines, we found both miR-99a and miR-100 to be downregulated 2-3 fold in the more advanced C4-2 line(Fig. 1b). Additionally, we decided to examine the expression of these microRNAs following IR in LnCAP cells and found it to be 2-3 fold upregulated 8 hours following IR, but was reduced to 1.4-1.8 fold by 24 hours following IR, indicating that there is a transient upregulation following IR(Fig. 1c). We were interested in determining whether expression of miR-99a and miR-100 correlated with radiation sensitivity of these cell lines and thus subjected them to ionizing radiation and performed clonogenic assays to determine recovery and survival of cells as colony forming units following DNA damage. We found that the more advanced C4-2 cancer cells displayed significantly greater survival following radiation than the LnCAP cells(Fig. 1d). Additionally we observed a 2 fold increase in survival of SK-BR-3 cells as compared to MCF7 when treated with 5gy

IR(Fig. 1e). These findings suggest a correlation between the expression level of miR-99a family members and radiation sensitivity of the cancer cell. We chose to further study the effect of this microRNA family on radiation sensitivity in the related C4-2 and LnCAP cell lines to minimize genetic differences contributing to varying phenotypes between cell lines. In order to determine if expression of miR-99a could sensitize cells to DNA damage, we introduced exogenous miR-99a into C4-2 cells, and found a significant reduction in clonogenic survival following radiation(Fig. 1f). These data suggest that the miR-99 family may regulate the sensitivity of cells to DNA damage and that loss of miR-99 family member expression may be a mechanism by which cancer cells can acquire resistance to DNA damaging agents.

miR-99a and siRNA of its target SNF2H reduce BRCA1 localization to sites of DNA damage.

We previously identified SNF2H as a direct target of miR-99a, and found it to regulate PSA expression in prostate cancer cells. Recent publications have reported that the chromatin remodeling factor SNF2H facilitates recruitment of DNA repair proteins to sites of DNA damage and is required for efficient homologous recombination and non-homologous end joining repair at DNA double strand breaks(Lan et al., 2010; Nakamura et al., 2011). In light of this, we determined to test if whether miR-99a could alter the efficiency of DNA repair by regulating the expression of SNF2H. To examine the effect of miR-99a on the recruitment of DNA repair proteins, we introduced miR-99a or siRNA targeting the 3' UTR of SNF2H into C4-2 cells and subjected them to gamma irradiation and then examined BRCA1 and pH2AX foci formation by immunofluorescence. Both miR-99a and siSNF2H greatly reduced the number of cells expressing intense BRCA1 IRIFs as well as the number of foci per cell(Fig. 2a, 2b). Neither treatment reduced pH2AX foci following IR. We also examined MDC1 and conjugated ubiquitin foci formation following IR, and saw no significant reduction in the number of these foci(Fig. S1), consistent with the mechanism that SNF2H facilitates BRCA1 recruitment downstream of the ubiquitination of H2B by RNF20 in a pH2AX independent manner.

Recruitment of DNA repair proteins is unaffected by miR99a in cells expressing non-targetable SNF2H.

To determine if SNF2H was directly responsible for the effect on BRCA1 recruitment with miR-99a treatment, we stably expressed the SNF2H ORF in C4-2 cells and examined BRCA1 foci in these cells following irradiation. We found that this non-targetable SNF2H rescued BRCA1 foci formation both after treatment with miR-99a as well as siSNF2(Fig. 2c, 2d). This evidence suggests that the effect of miR-99a on BRCA1 recruitment is through its downregulation of SNF2H.

miR-99a reduces the rate of DNA repair through downregulation of SNF2H

To assess the effect of miR-99a on the efficiency of DNA repair, we transfected C4-2 cells with miR-99a and siSNF2H and performed neutral comet assays following treatment with IR. With the control

siRNA oligo, we observed that the majority of DNA damage was repaired within 60 minutes of treatment with IRIn the presence of miR-99a or siSNF2H, the DNA damage persisted, which resulting in a 3x greater tail moment than the control at 60 minutes following irradiation(Fig. 3a, 3b).. However, when we stably expressed the SNF2H ORF using a retroviral vector in these cells, they became resistant to treatment with miR-99a or siSNF2H, and the rate of DNA repair was rescued(Fig. 3c).

miR-99a, miR-100 and siSNF2H block efficient repair of double strand breaks by homologous recombination.

Having observed the effect of miR-99a and miR-100 on BRCA1 foci formation, we needed to determine whether they could reduce the rate of successful homologous recombination following DNA damage. We transfected miR-99a, miR-100 and siSNF2H into DR13-9 HeLa cells, which contain two partial GFP cassettes containing I-sce1 sites. Fusion of the full lenth GFP occurs following successful homologous recombination after I-sce1 expression, resulting in GFP expression. In the presence of miR-99a or siSNF2H, we observed a 40 percent reduction in the level of GFP expression(Fig. 4a). Transfection with siRNA targeting ATM and BRCA1 resulted in 70 and 90 percent respective reductions in HR efficiency. These data show that miR-99a, miR-100 and siSNF2H can reduce the efficiency of homologous recombination following double strand breaks, which correlates with the decreased recruitment of BRCA1 to sites of DNA damage.

miR-99a, miR-100 and siSNF2H block efficient non-homologous end joining type repair of double strand breaks.

SNF2H has also been previously shown to be important for the recruitment of Ku70/80 to sites of DNA damage, facilitating efficient repair of double strand breaks by non-homologous end joining. To determine if miR-99a and miR-100 could also reduce the efficiency of NHEJ we utilized a reporter assay that generates expression of DS-Red following successful non-homologous end joining after I-sce1 generated double strand breaks. When we introduced siSNF2H, we observed a 60 percent reduction in DS-Red expression, and a 50 percent reduction with exogenous miR-99a or miR-100(Fig. 4b). siRNA targeting ATM also displays a significant reduction successful NHEJ whereas siBRCA1 showed no significant reduction in NHEJ type repair. This suggests that miR-99a/100 can reduce the efficiency of multiple pathways of double strand break repair through its regulation of SNF2H.

Induction of miR-99a and miR-100 following ionizing radiation reduces the efficiency of DNA repair when confronted with multiple rounds of DNA damage.

Having observed that miR-99a and miR-100 are both transiently induced following DNA damage and can reduce the efficiency of DNA repair when introduced into cells, we hypothesized that when confronted with multiple rounds of DNA damage, cells may become less efficient at DNA repair due to upregulation of the miR-99 family. To determine if cells less efficiently form DNA repair foci after

miR-99a/miR-100 upregulation, we subjected LnCAP cells to 2gy of ionizing radiation and then incubated them for 24 hours. We then subjected the cells to an additional 2gy of IR and examined BRCA1 foci formation 1 hour following the second treatment. We found that after 2 gy IR, 80 percent of the cells formed strong BRCA1 foci. However, 24 hours following the initial DNA damage, when treated with another round of radiation, only 40 percent of cells formed strong BRCA1 foci(Fig. 5b, 5c). To determine if this effect was dependent on the induction of the miR-99 family, we introduced 2'-O-methyl antisense oligonucleotide inhibitors of miR-99a and miR-100 into the cells at the time of the initial radiation treatment to block their induction. When treated with the inhibitors, we observed no change in BRCA1 foci formation between the two rounds of IR treatment, indicating that induction of miR-99a and miR-100 were responsible for the reduction in BRCA1 recruitment to double strand break sites after multiple rounds of DNA damage(Fig. 5b, 5c).

We have identified a microRNA family which is upregulated in response to ionizing radiation and can reduce cells ability to repair damaged DNA. Through regulation of SNF2H, the miR-99 family can affect the efficiency of DNA repair by multiple mechanisms. The expression of this microRNA appears to be lost in more advanced cancers, and positively correlates with radiation sensitivity in the cell lines we tested. Loss of this microRNA may represent a mechanism by which cancer cells can acquire resistance to DNA damage by allowing efficient repair to continue after multiple mutagenic insults. Induction of this microRNA in more normal cells and less aggressive cancers appears to function as a switch whereby cells exposed to a single large dose of DNA damage tend to efficiently repair their DNA, following which the mirR-99 family is induced and repair mechanisms are downregulated, shifting cell fate away from repair and survival and towards apoptosis if multiple rounds of DNA damage are experienced within a short time. This is further supported by recent findings that the miR-99 family targets pro-survival proteins IGF-1R and mTOR(Doghman et al., 2010), indicating that there are multiple pathways the miR-99 family targets that would presumably have an effect on the cells survival following DNA damage. Since loss of miR-99 family expression in more advanced cancer cells seems to correlate with radioresistance, and re-introduction decreases survival following radiation, it may be useful as a radiosensitizing agent when in vivo delivery of microRNAs becomes a therapeutic reality.

Materials and Methods

Cell Culture

Human prostate cancer cell lines LnCAP and C4-2, as well as human breast cancer cell lines MCF7 and SK-BR-3 were obtained from ATCC. HeLa DR13-9 cells were obtained from the Parvin laboratory. NHEJ-DSRed 293B cells were obtained from the Valerie laboratory through the Larner laboratory. LnCAP and C4-2 were grown in RPMI 1640 medium supplemented with 10% FBS. MCF7, HeLa

DR13-9 and NHEJ-DSRed 293B cells were cultured in DMEM with 10% FBS, and SK-BR-3 cells were cultured in McCoy's 5a medium with 10% FBS. All cell lines were cultured under normal conditions.

Clonogenic assays

Cells were transfected in 10 cm dishes with 20 picomoles of miR-99a duplex or siRNA against firefly luciferase as a control. The oligos were incubated for 30 minutes with 8uL Lipofectamine RNAimax per plate in 2mL Optimem before mixing with trypsinized cells and plated at a 20% confluence. Plates were incubated for 48 hours before seeding to 6-well plates in the presence of 20nM duplex oligo at increasing seeding densities corresponding with the planned dose of radiation. 6-well plates were irradiated in a Shepherd Mark 1 Cs-137 irradiator. Cells were incubated until colonies arising from single cells reached 25 cells or more, with media changed every 72 hours. Colonies were counted, and clonogenic survival was calculated by dividing the number of colonies at a given dose by the number of colonies in the un-irradiated control samples, and multiplied by the seeding density coefficient.

miRNA expression analysis

RNA was isolated from cells using Trizol extraction(Invitrogen). For microarray analysis, RNA was further purified using RNAeasy RNA cleanup kit(Qiagen), and sent to Exiqon for hybridization to miRCURY LNA V10.0 microRNA analysis microarrays. For qPCR validation, Poly A tailing and cDNA preparation of mature microRNAs was performed using the NCODE miRNA amplification system(Invitrogen). qPCR amplification was performed using forward primers identical to the mature miRNA sequence, and NCODE universal reverse primers with Sybr Green ER(Invitrogen). Expression was normalized to u6snRNA, which used the primer sequence 5′-CTGCGCAAGGATGACACGCA-3′.

Immunofluorescence

Cells were plated on glass cover slips in the presence of 20nM siRNA duplex. After 72 hours, the coverslips were irradiated with 5gy IR. After 60 minutes, the cover slips were fixed with 4% formaldehyde in PBS, and permeabilized in 0.5% Triton X-100 in PBS. Coverslips were blocked in 5% goat serum, or 5% donkey serum if primary antibody is of goat origin(MDC1). Coverslips were incubated at room temperature with primary antibody for 1 hour, and Alexa 488 or 549 conjugated secondary antibody for 1 hour, with three TBS washes following each antibody incubation. Coverslips were then mounted with Vectashielf mounting solution(Vector Laboratories). Images were collected at equal exposures and foci were counted manually using ImageJ. Antibodies used were BRCA1 D-9(Santa Cruz), pH2AX (Cell signaling, Millipore), MDC1 C-20(Santa Cruz), Anti conjugated ubiquitin FK2 (Millipore). pH2AX primary antibodies was used at a 1:200 concentration in 5% goat serum, MDC1 antibody was used at a 1:100 concentration in 5% donkey serum, all other antibodies were used at a 1:100 concentration in 5% goat serum.

Homologous Recombination/NHEJ assay

20% confluent HeLa DR13-9 cells and NHEJ-DSRed 293B cells were treated transfected with 20nM siRNA using Lipofectamine RNAimax in 10 cm plates. After 24 hours, each plate was transfected with 20ug I-sce1 plasmid with Lipofectamine 2000. After another 48 hours, cells were trypsinized, resuspended in media and analyzed by FACS for GFP or DSRed expression.

Comet Assay

Cells were transfected for 72 hours with 20nM siRNA oligo in 6cm plates, followed by 10gy irradiation. Cells were then trypsinized following irradiation and resuspended in ice cold PBS, incubated on ice until all time points were collected. Neutral comet assays were then performed using the Trevigen Comet assay kit standard protocol.

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Figure Legends

Table 1. Expression changes following IR in microRNAs differentially expressed between MCF7 and SK-BR-3 breast cancer cell lines. Microarray analysis of microRNA expression shows MiR-99a and miR-100 are both overexpressed in MCF7 and upregulated 24 hours following 5gy of ionizing radiation.

Figure 1. miR99 family are downregulated in more radioresistant breast and prostate cancer, transiently upregulated following DNA damage and can confer radiation sensitivity to cells. (A) miR-99a and miR-100 are overexpressed in MCF7 breast cancer cells compared to SK-BR-3 breast cancer cells. (B) miR-99a and mir-100 are downregulated in C4-2 androgen independent prostate cancer cells compared to the

androgen dependent LnCAP parental cell line. (C) miR-99a and miR-100 are transiently upregulated in LnCAP cells following treatment with 5gy ionizing radiation. (D) Clonogenic assay of C4-2 and LnCAP cells following a dose titration of IR. C4-2 cells are more resistant to cell death following radiation. (E) Clonogenic survival of MCF-7 and SK-BR-3 following 5gy ionizing radiation. SK-BR-3 cells display greater survival at this dose of IR than MCF-7 cells. (F) miR-99a sensitizes C4-2 cells to ionizing radiation. Transfection of C4-2 cells with double stranded miR-99a mimic reduces cell survival by clonogenic assay at multiple doses of radiation.

Figure 2. miR-99a reduces BRCA1 foci formation following IR through its target SNF2H. (A) miR-99a and siRNA directed against SNF2H reduce BRCA1 foci formation following IR. Cells were treated with 5gy IR following a 72 hour incubation with either ds-miR-99a or siSNF2H, BRCA1 foci formation was visualized by immunofluorescence 1 hour after IR treatment. (B) Quantitation of BRCA1 foci formation. The number of cells expressing BRCA1 foci following IR is reduced by ds-miR-99a or siSNF2H treatment, but p-H2AX foci formation is unaffected. (C) BRCA1 foci formation is unaffected by treatment with ds-miR-99a or siSNF2H in cells expressing a non-targetable form of SNF2H. The siRNA against SNF2H as well as miR-99a target the 3'UTR of the SNF2H transcript, and retroviral expression of the SNF2H open reading frame rescues BRCA1 foci formation following IR. (D) Quantitation of cells expressing BRCA1 foci in C4-2 cells expressing non-targetable SNF2H.

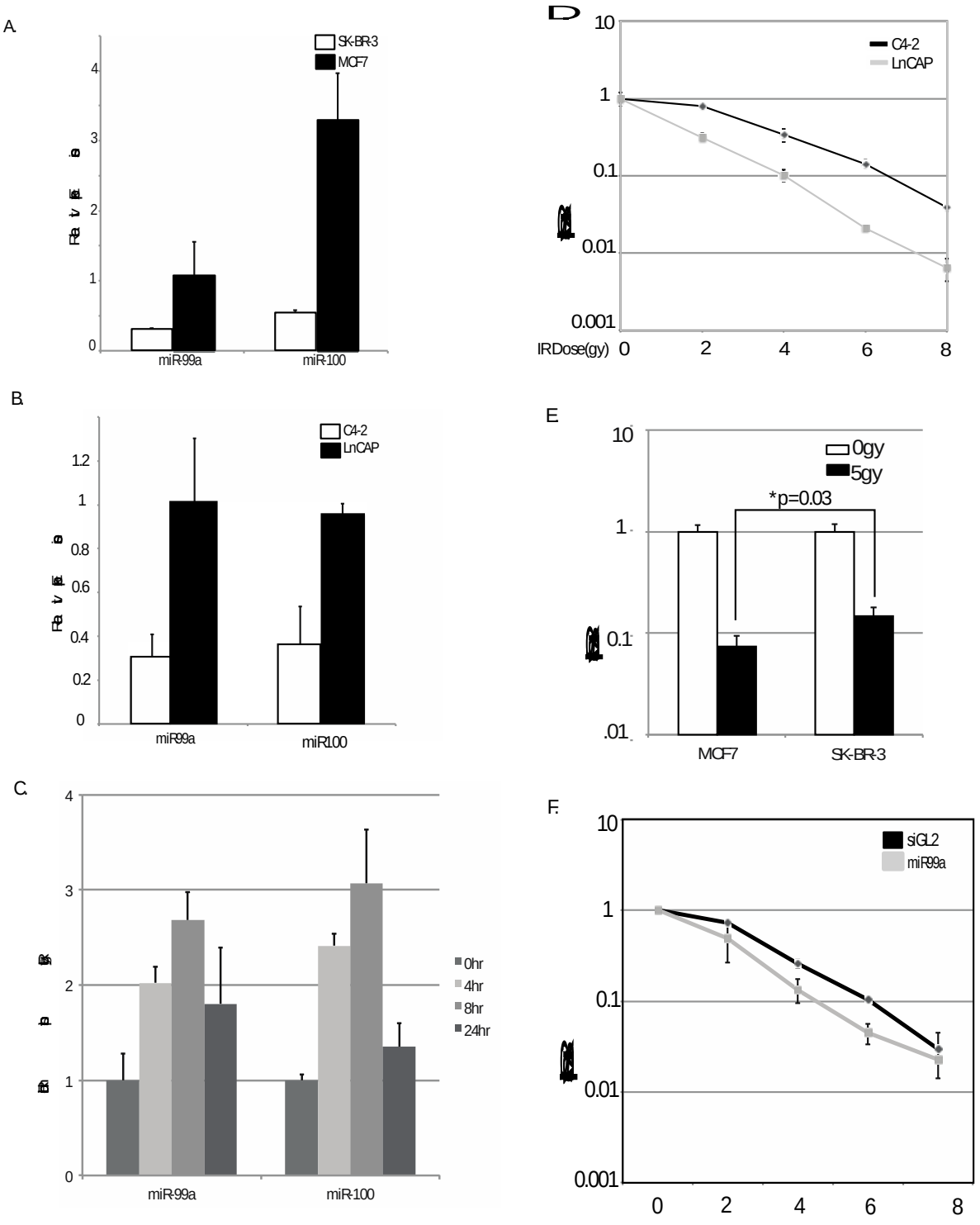
Figure 3. miR-99a slows the kinetics of DNA repair following IR through SNF2H. (A) The rate of DNA repair in C4-2 cells is reduced by introduction of ds-miR-99a or siSNF2H as shown by the persistence of a comet tail at 60' and 120' following IR. DNA damage was measured by neutral comet assay at time points following 10gy IR. (B) ds-miR-99a and siSNF2H significantly decrease the rate of DNA repair as shown by the increased comet tail moment compared to control cells at 30' and 60' following IR. (C) Cells expressing non-targetable SNF2H display similar rates of DNA repair when transfected with control siRNA, ds-miR-99a or siSNF2H, indicating that the reduction in rate of DNA repair normally seen in the presence of miR-99a occurs through its effect on SNF2H expression.

Figure 4. The efficiency of homologous recombination and non-homologous end joining are reduced by expression of ds-miR-99a, ds-miR-100 and siSNF2H. (A) I-sce1 transfected DR13-9 HeLa cells show a reduction in the number of cells positive for GFP expression representing effective HR in the presence of exogenous miR-99a, miR-100, or siSNF2H. siRNA targeting ATM and BRCA1 also significantly reduced HR repair in this system. (B) 293B cells expressing a DS-Red NHEJ reporter displayed a decrease in effective NHEJ when I-sce1 expression was induced in the presence of ds-miR-99a, ds-miR-

100 or siSNF2H. siRNA targeting ATM reduced the efficiency of NHEJ, whereas siBRCA1 did not significantly reduce NHEJ efficiency.

Figure 5. Induction of miR-99a/miR-100 following radiation in LnCAP cells decreases the efficiency of repair following additional rounds of radiation. (A) Diagram of the effect of miR-99a/miR-100 induction following multiple rounds of IR. Cells were treated with 2gy IR and then allowed to incubate for 24 hours prior to treatment with another round of 2gy IR. miR-99a/miR-100 induction was blocked by introduction of 2'-O-methyl antisense oligos targeting mir-99a and miR-100 at the time of the first treatment. (B) BRCA1 foci formation following IR is reduced after multiple rounds of treatment in a miR-99a/miR-100 dependent manner. (C) Quantitation of BRCA foci formation over multiple rounds of IR treatment. With the control oligo, after 2gy IR, 80% of LnCAP cells showed strong BRCA1 foci formation, which was reduced to 40% when treated with an additional round of IR 24 hours later. When incubated with 2'-O-methyl anti-miR-99a and anti-miR-100 at the time of the first treatment, there was no change in the efficiency of BRCA1 foci formation after the second treatment 24 hours later.

Figure 1.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MCF7
miR-99a
miR-100
B
C4-2
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miR100
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8hr
24hr
miR-99a
miR-100
D
C4-2
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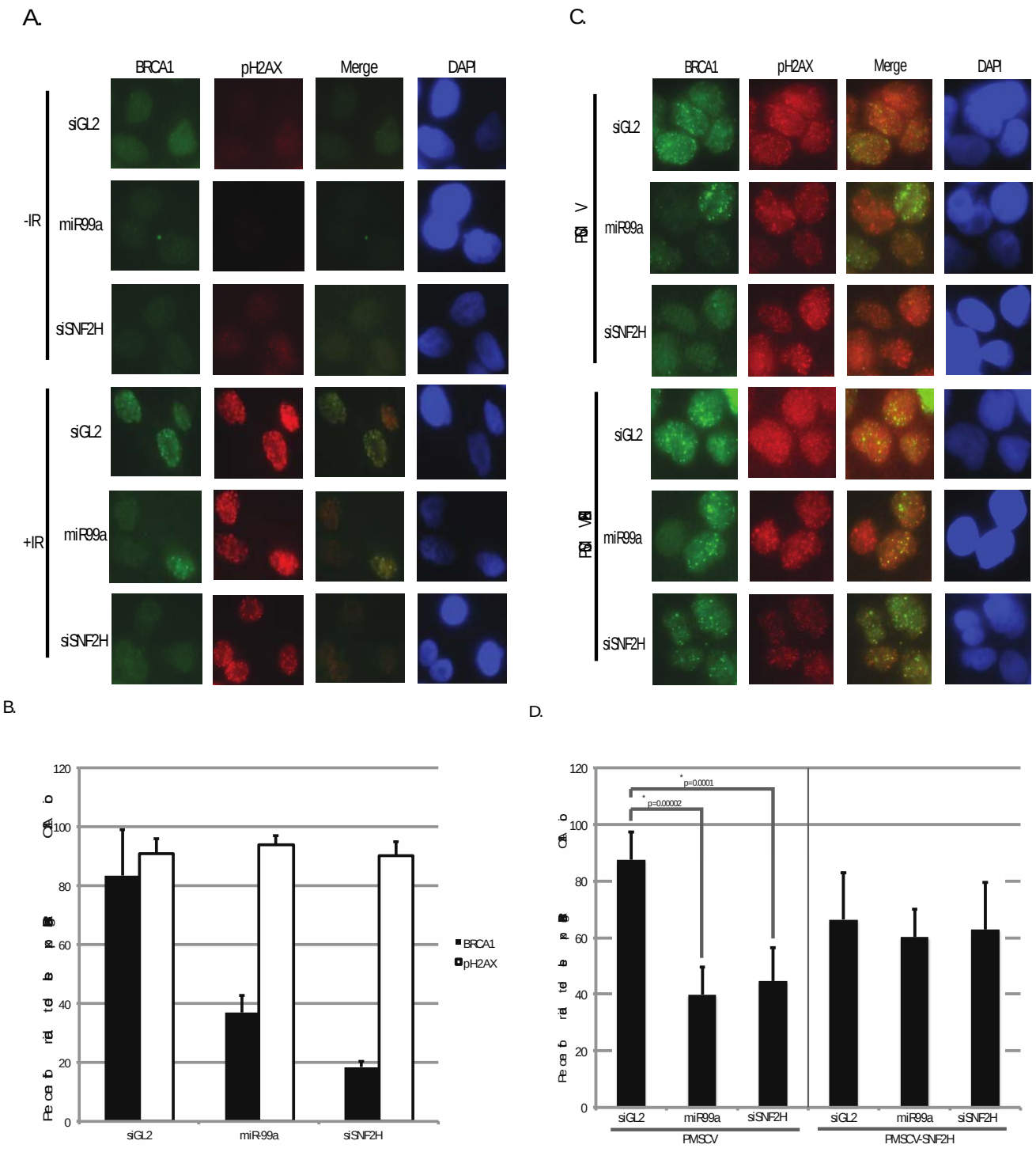
miR99a

Figure 2.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B.
D.
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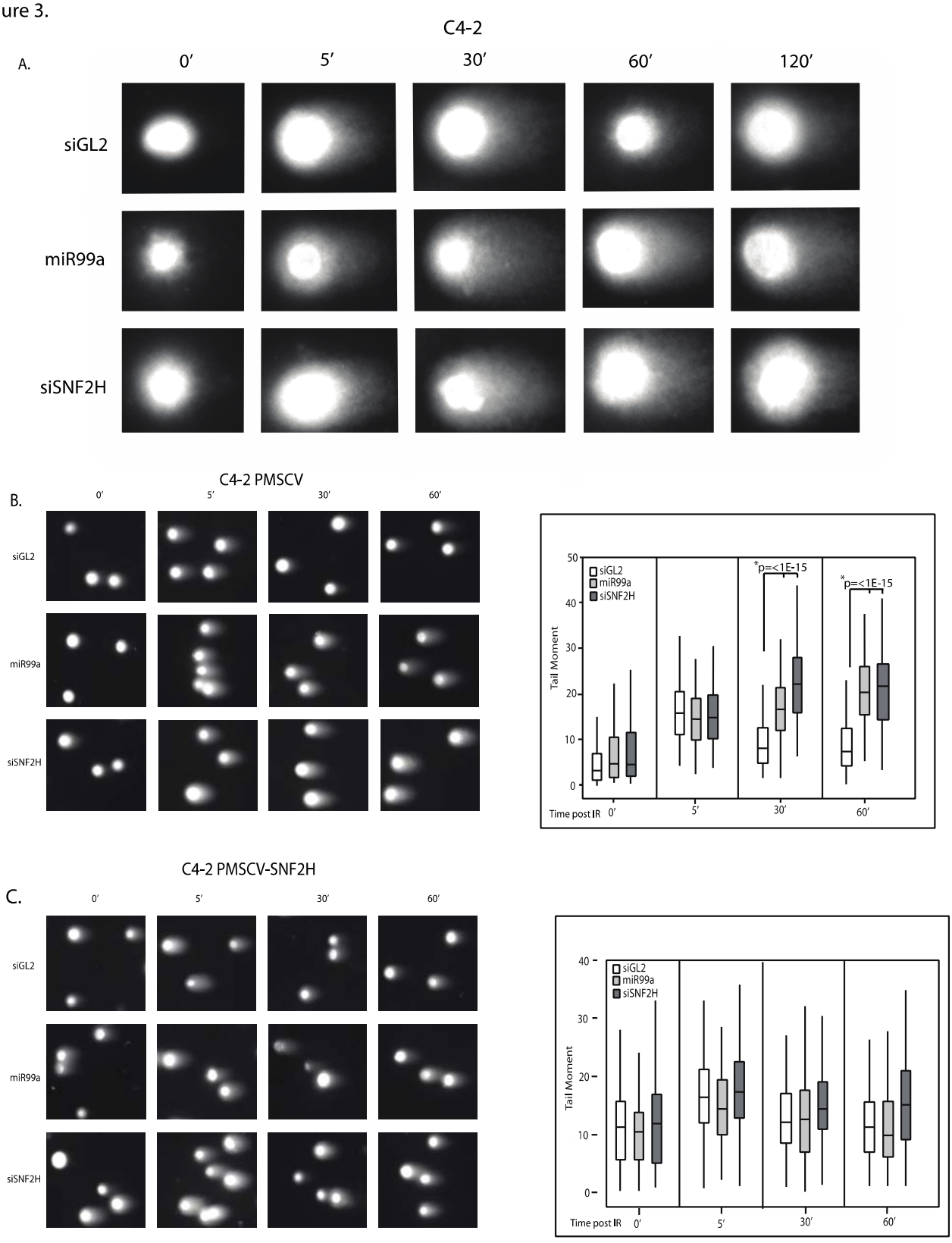
Figure 3.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siGL2
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siSNF2H
C4-2 PMSCV
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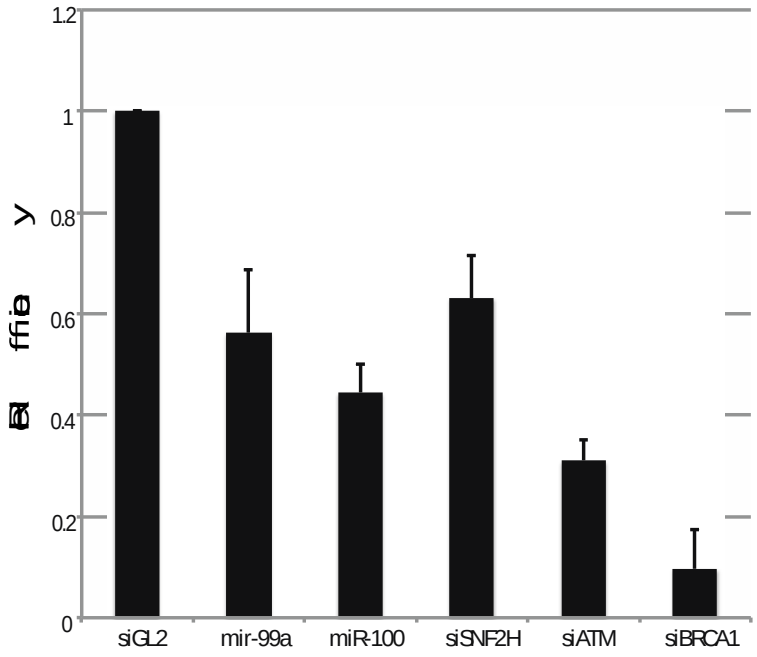
Figure 4.

A.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miR-100
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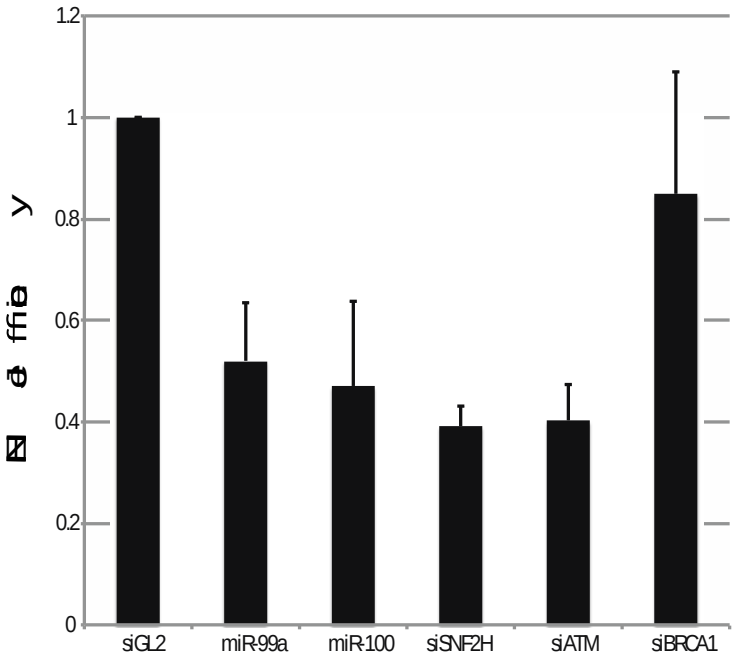
siATM
siBRCA1

B.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0.8
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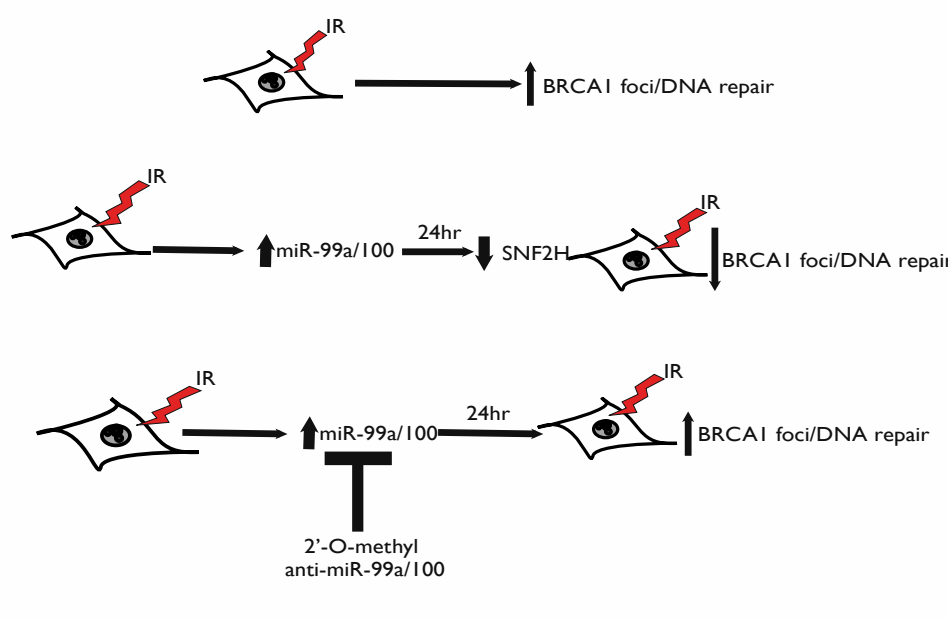
Figure 5.

A.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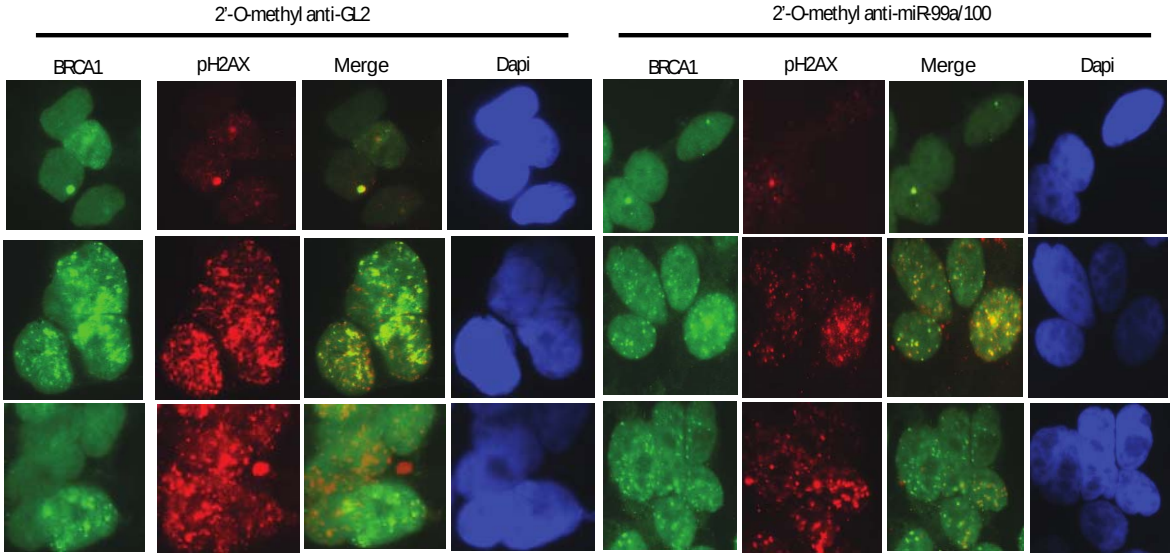
BRCA1 foci/DNA repair
IR
miR-99a/100
24hr
SNF2H
IR
BRCA1 foci/DNA repair
IR
miR-99a/100
24hr
IR
BRCA1 foci/DNA repair
2'-O-methyl
anti-miR-99a/100

B.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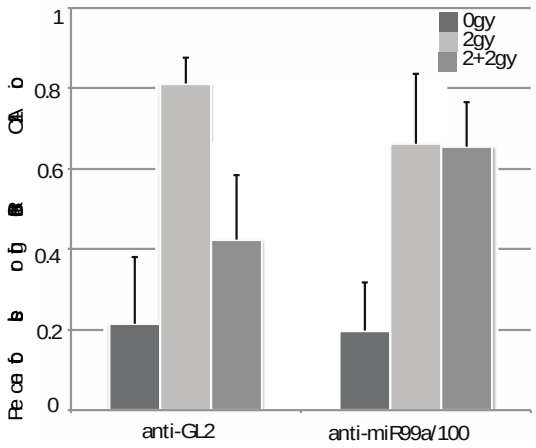
BRCA1
pH2AX
Merge
Dapi
BRCA1
pH2AX
Merge
Dapi
0gy
2gy
2gy
+
2gy

C.


1
0.8
0.6
0.4
0.2
0
0gy
2gy
2+2gy
anti-GL2
anti-miR99a/100

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Adam Mueller	POSITION TITLE Graduate Student
eRA COMMONS USER NAME	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Harford Community College	AS	1999	Biological Sciences
UMBC	BS	2002	Biological Sciences
UMBC	MS	2003	Applied Molecular Biology
University of Virginia School of Medicine	MD/PhD (ongoing)	2004-Present	Biochemistry and Molecular Genetics/Medicine

Please refer to the application instructions in order to complete sections A, B, and C of the Biographical Sketch.

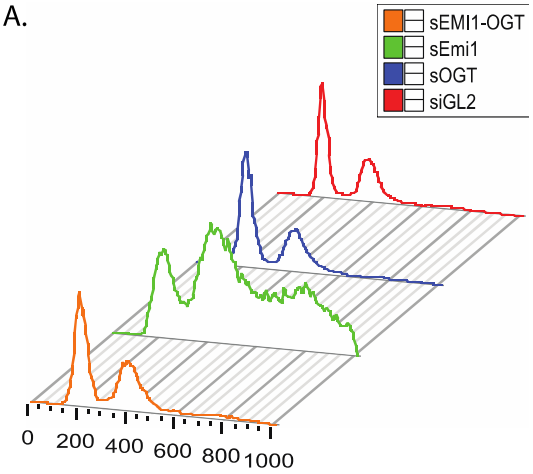
Research Positions:
1999-2000: Student Research Contractor, Biomaterials, Aberdeen Proving Grounds, Edgewood, Maryland. Study of bacterial enzymatic digestion of V and G type nerve agents. PI: Ilya Elashvili
2002-2003: Graduate Student: Applied Molecular Biology Lab, UMBC, Inducing expression of MHC class II antigen presenting molecules in breast cancer cells. Lab Manager: Julie Wolf, PI: Suzanne Rosenberg.
2004: Rotation Graduate Student, UVA, Michael Weber Lab. MAP kinase signaling in SKOV3 ovarian cancer cells.
2005: Rotation Graduate Student, UVA, Isa Hussaini Lab. PKC signaling in Glioblastoma multiforme.
2005-Present: Graduate Student, UVA, Anindya Dutta Lab. Effect of statins on progression of breast cancer, as well as screening of growth essential genes in normal breast tissue.

Awards and Honors:
1996: HCC Alfred C' O'Connell Scholarship
1999: HCC Academic Achievement Award
1999: Maryland Burgee Board of Regents Scholarship
2004: University of Virginia Graduate School of Arts and Sciences Fellowship
2005: NIH Cancer Training Grant
Member Phi Theta Kappa Honors Society (Treasurer Rho Beta 1998)
Member Golden Key Honors Society
Member Phi Kapp Phi Honors Society

Non-Research Positions and Activities:
2000-2001: Assistant Coach. Joppatowne Tigersharks Swim Team.
2003: Associate Game Designer/Web Administrator. GRS Games/Gentle Revolution Press. Supervison: Victoria Kinnear
2002: President, 2003-2004 Assitant Instructor. UMBC Jujitsu Club.
2006-2007. Instructor, UVA Brazilian Jiu-Jitsu club.

Supporting Materials:

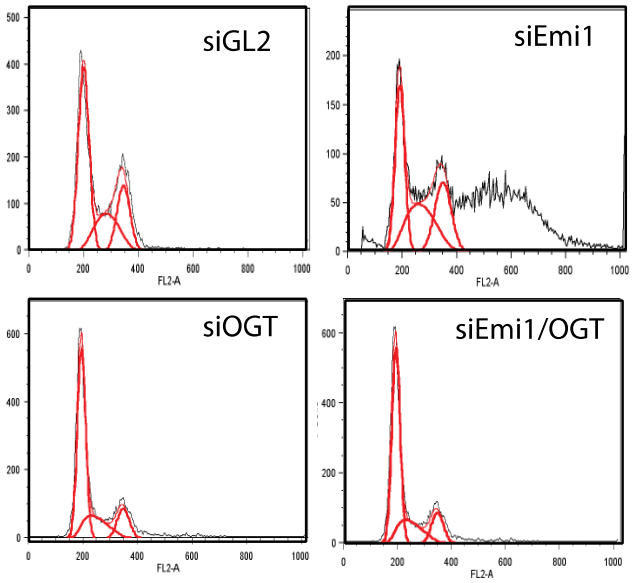
A.



MCF10a

Treatment	%Rereplication
siGL2	7
siOGT	8
siEMI1	50
siEMI1/OGT	11

B.



MCF7

Treatment	%Rereplication
siGL2	2
siOGT	10
siEMI1	50
siEMI1/OGT	10

C.

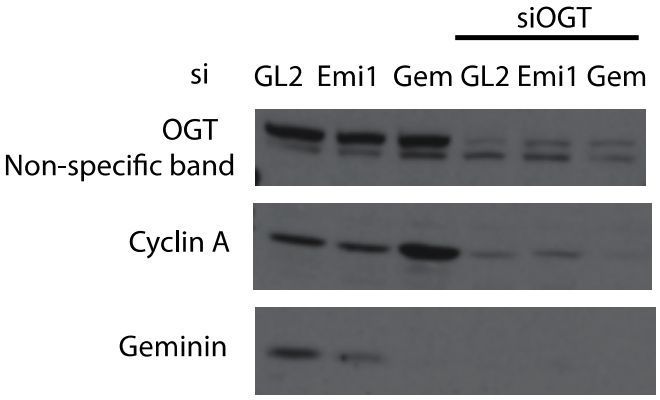


Figure 1. Cell cycle profile and %re-replication in MCF10a(A), and MCF7(B) cells following siEmi1 and siOGT treatment. Western blots for expression of OGT, Cyclin A and Geminin(C).

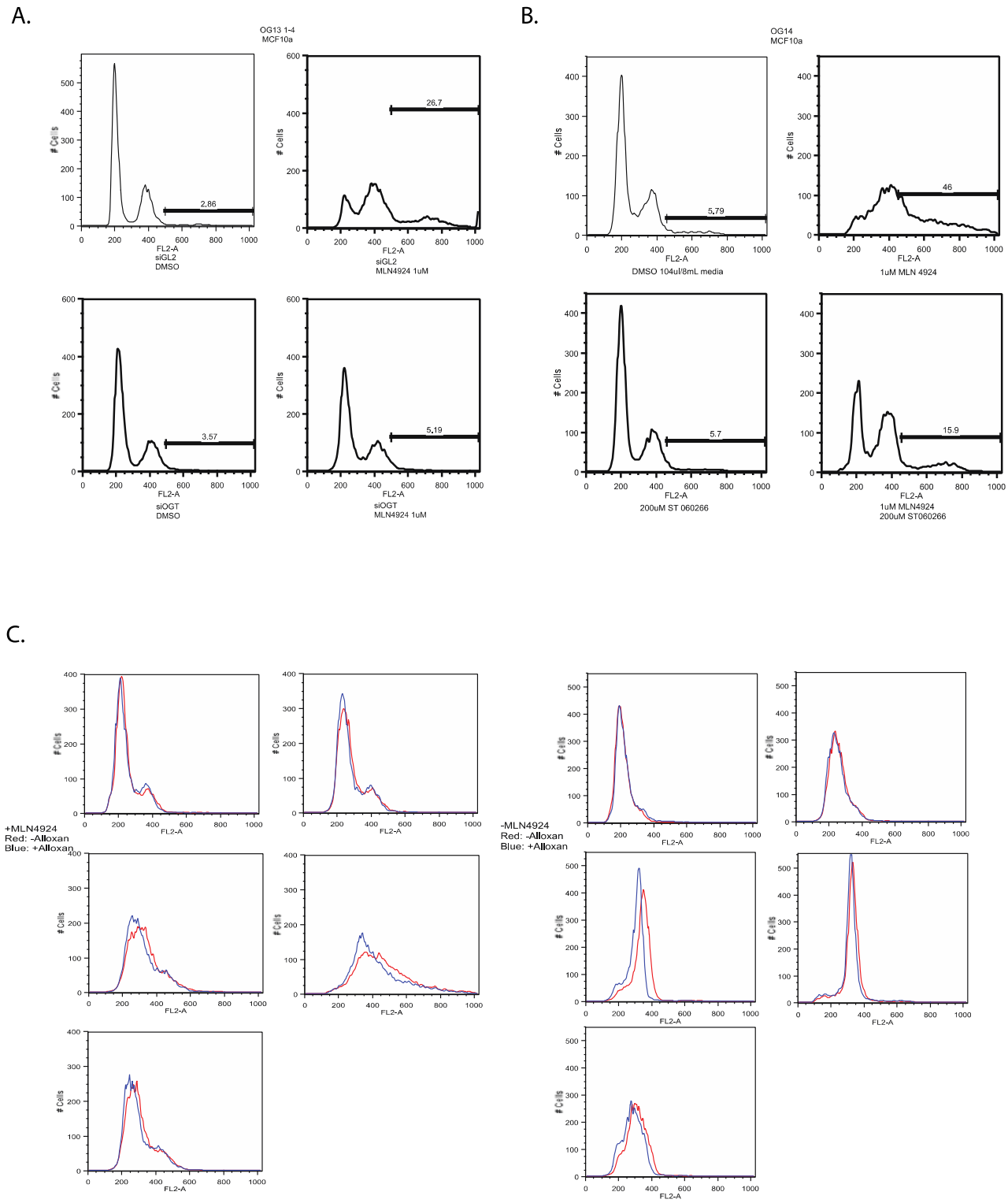
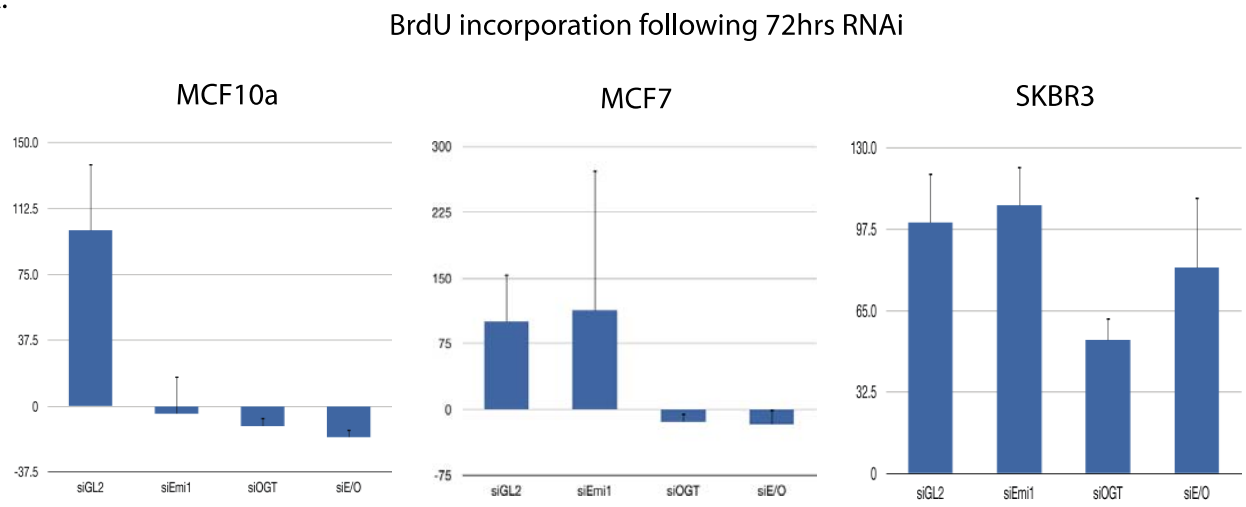


Figure 2. Cell cycle profile and %re-replication of MCF10a cells following siOGT and MLN4924 treatment(A). Cell cycle profile and %re-replication following treatment with MLN4924 and OGT inhibitor ST060266(B). Cell cycle profile of HCT116 cells following double thymidine block and release with MLN4924 and OGT inhibition by Alloxan(C), red=DMSO, blue=Alloxan, +MLN left panel, -MLN right panel.

A.



B.

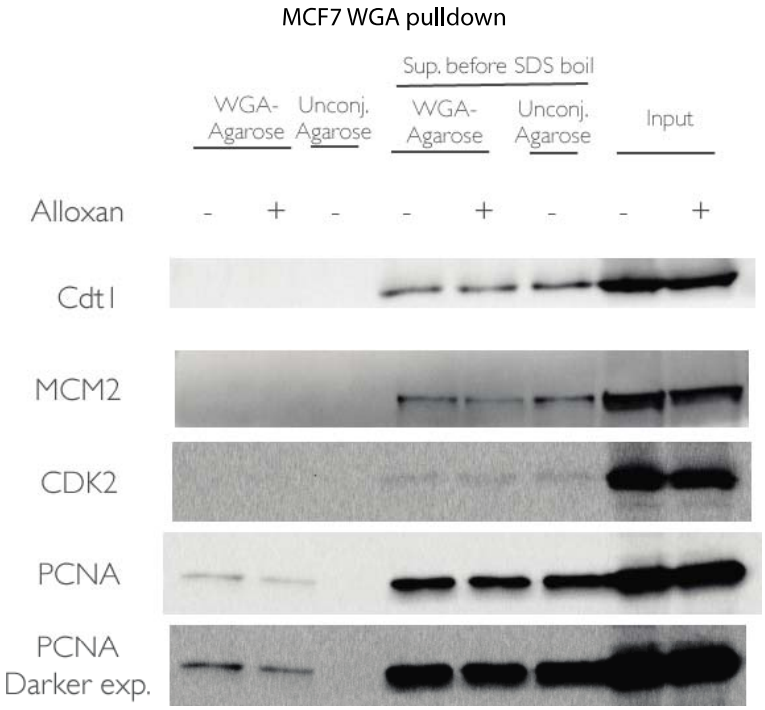
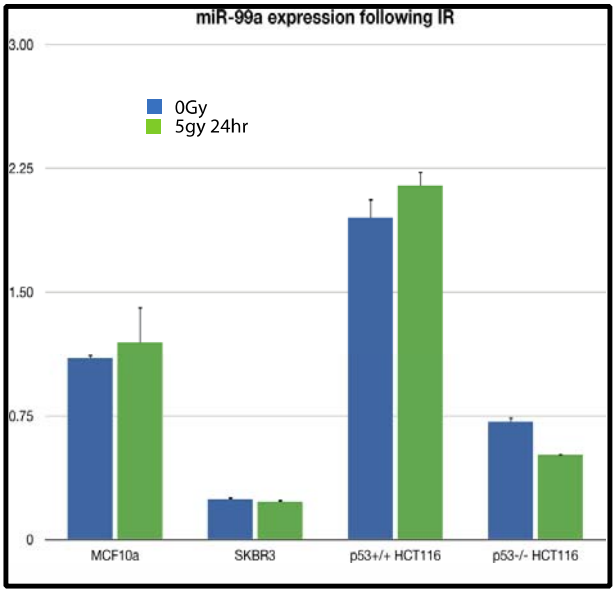
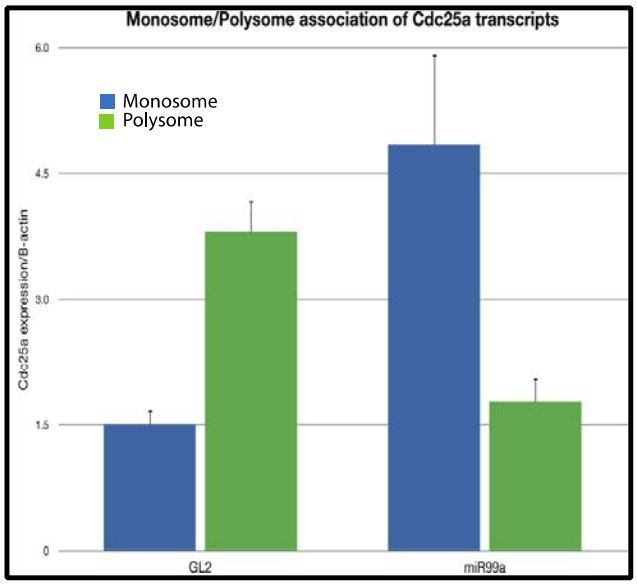


Figure 3. BrdU incorporation in MCF10a, MCF7 and SKBR3 following siEmi1, siOGT(A). WGA pulldown western blots for DNA replication and S-phase entry cell cycle proteins(B).

A.



B.



C. MCF10a 10gy IR time course 3H/14C inc.

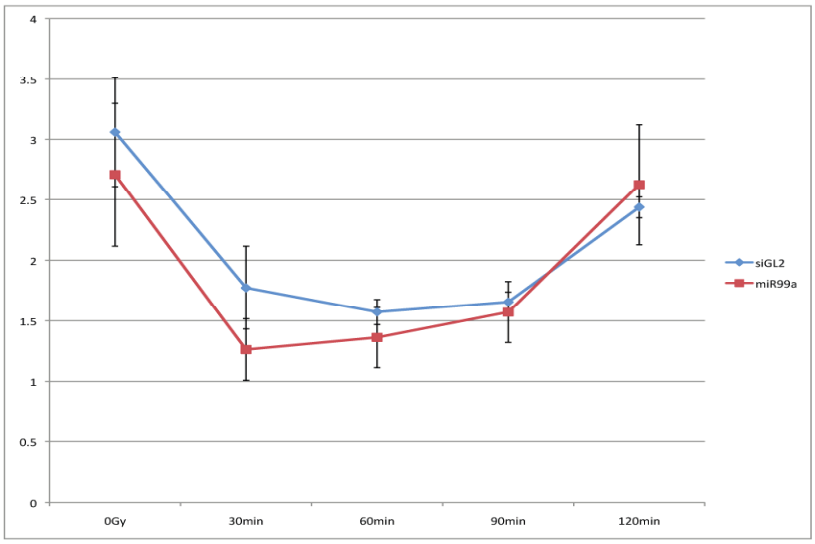
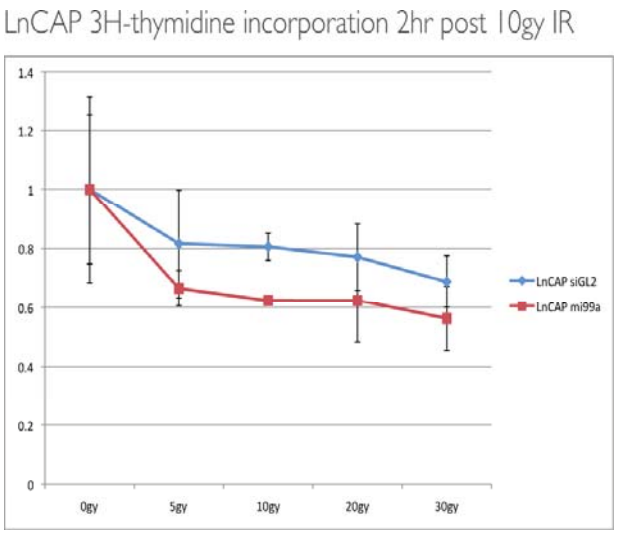
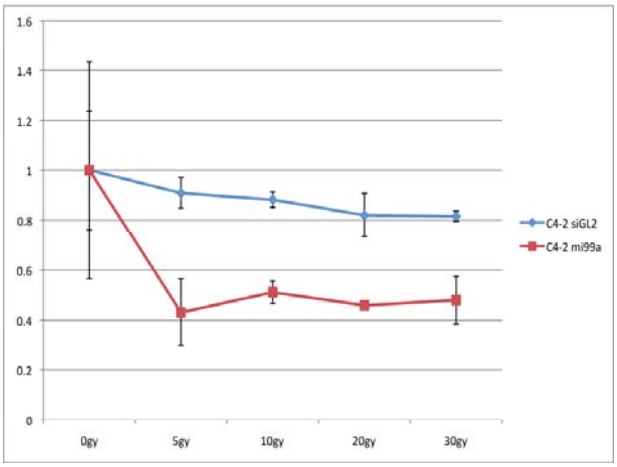


Figure 4. Expression of miR99a in breast and colon cancer cell lines(A). Association of Cdc25a with ribosomal fractions following miR99a introduction(B). 3H-Thymidine incorporation time course following IR in MCF10a(C).

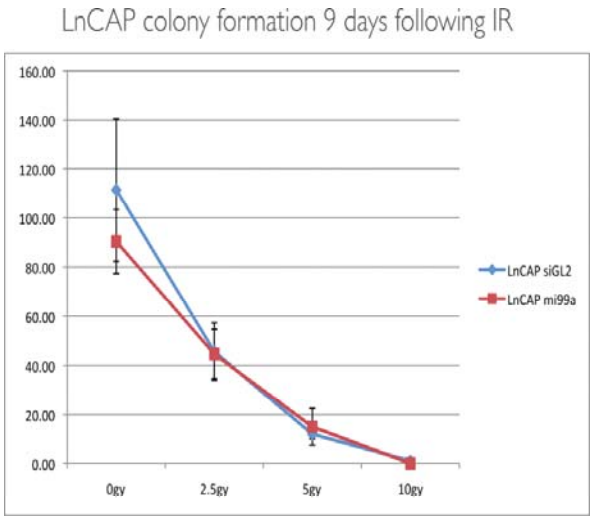
A.



B.



C.



D.

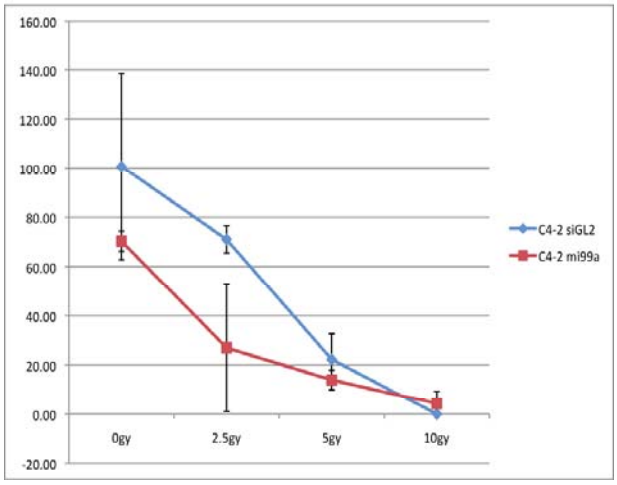


Figure 5. Thymidine incorporation following IR dose titration in LnCAP and C4-2 cells(A,B). C4-2 clonogenic survival +/- miR99a following IR(C,D).

Figure 6

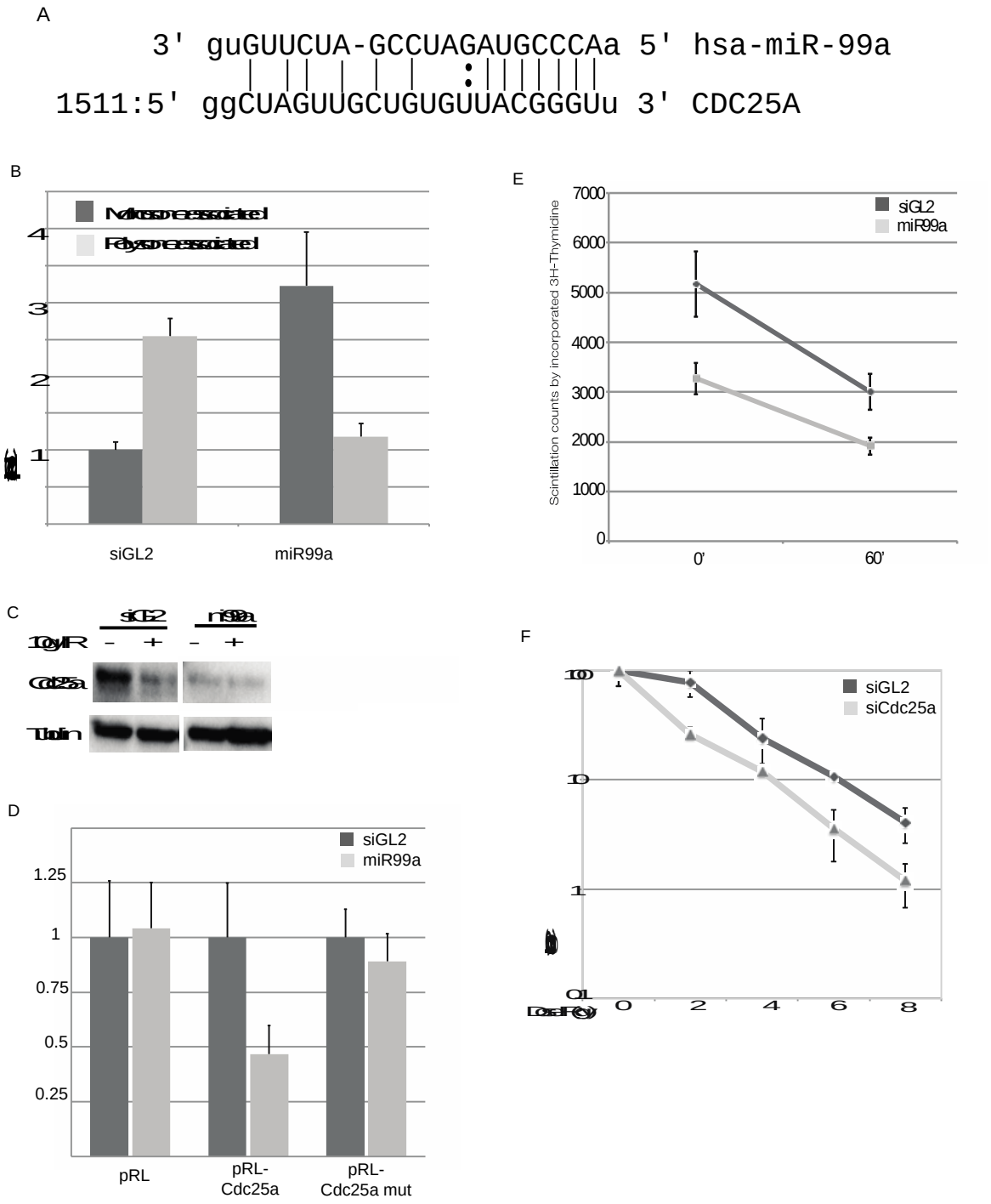


Figure 6. Cdc25a is a direct target of miR99a. (A) miR-99a target sequence of Cdc25a 3'UTR. (B) Polysome/monotome associated expression of Cdc25a transcript with miR-99a introduction.(C) Cdc25a protein expression following introduction of exogenous miR-99a +/- ionizing radiation. (D) Cdc25a 3'UTR coupled luciferase activity following miR-99a introduction. (E) Thymidine incorporation following miR-99a introduction. (F) Clonogenic survival following IR with Cdc25a siRNA.